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Cyclosporine withdrawal and mycophenolate mofetil treatment effects on the progression of chronic cyclosporine nephrotoxicity

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Cyclosporine withdrawal and mycophenolate mofetil treatment effects on the progression of chronic cyclosporine nephrotoxicity.

Background. Recent clinical trials of mycophenolate mofetil (MMF) in chronic allograft nephropathy (CAN) demonstrated that the dose of cyclosporine A (CsA) is one of the critical factors in determining graft function in CAN, but the effect of MMF on chronic CsA nephropathy is undetermined. We undertook this study to evaluate the effect of MMF on CsA-induced nephrotoxicity in an animal model of chronic CsA nephropathy.

Methods. In the first experiment, Sprague-Dawley rats on a low-salt diet were treated with CsA (7.5 mg/kg per day) for 10 weeks, or were treated with CsA for five weeks and then MMF (20 mg/kg per day) was administered five weeks later. In the second experiment, rats were treated with CsA for five weeks, and CsA was then withdrawn for five weeks with or without MMF treatment. Renal function, histologic parameters (tubulointerstitial fibrosis, arteriolopathy, ED-1–positive cells, renin-positive glomeruli, TUNEL-positive cells) and the expression of osteopontin and transforming growth factor (TGF)-β1 mRNA expressions were compared for different treatment groups.

Results. CsA-treated rats showed decreased renal function and increased histologic parameters compared with the vehicle (VH)-treated rats. The addition of MMF did not improve these parameters compared with the CsA-treated rats. With CsA withdrawal, renal function and histologic parameters were significantly improved compared with the CsA-treated rats, and MMF treatment after CsA withdrawal further improved the histologic parameters. At the molecular level, the addition of MMF did not decrease the expression of osteopontin and transforming growth factor-β1 (TGF-β1) mRNAs, which were increased in the CsA-treated rat kidney. With CsA withdrawal, the expression of both mRNAs was significantly decreased compared with the CsA group, and a further decrease was observed with MMF treatment after CsA was withdrawn.

Conclusion. The combined treatment of CsA and MMF does not prevent the development of chronic CsA nephrotoxicity, but MMF treatment after CsA withdrawal does improve chronic CsA nephrotoxicity. This finding provides a rationale for MMF treatment in chronic CsA nephrotoxicity.

Key words: chronic allograft nephropathy, transplantation, nephrotoxicity, immunosuppression, progressive renal failure.

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Most immunosuppressive drug regimens depend on the cyclosporine (CsA) or FK506, even though nephrotoxicity is the major dose-limiting adverse effect for both drugs [1, 2]. Long-term administration of CsA causes progressive renal failure and irreversible renal striped interstitial fibrosis, tubular atrophy, and hyalinosis of the afferent arteriole. In the clinical context, chronic CsA nephrotoxicity is difficult to distinguish from chronic allograft nephropathy (CAN) [3], but its role in CAN may affect nearly 6% of the total renal transplant population [4].

Animal model of chronic CsA nephrotoxicity was established from the observation that sodium depletion exacerbates CsA nephrotoxicity [5, 6]. In this model, CsA treatment in rats on a low salt diet (LSD) induced a histological feature similar to that described in patients on long-term CsA therapy [7]. With this model, it is known that CsA stimulates the intrarenal renin-angiotensin system [8], transforming growth factor (TGF)-β1 production [9], and inflammatory response consisting of increased macrophage infiltration and osteopontin expression [10]. In addition, CsA treatment increases the expression of pro-apoptotic genes [11], and this is mediated by activation of angiotensin II and decreased production of nitric oxide [12].

Mycophenolate mofetil (MMF) has been used in solid-organ transplants as an effective adjunctive immunosuppressive agent. MMF selectively suppresses the proliferation of T and B lymphocytes, inhibits the formation of antibodies by inhibiting purine nucleotide synthesis, and

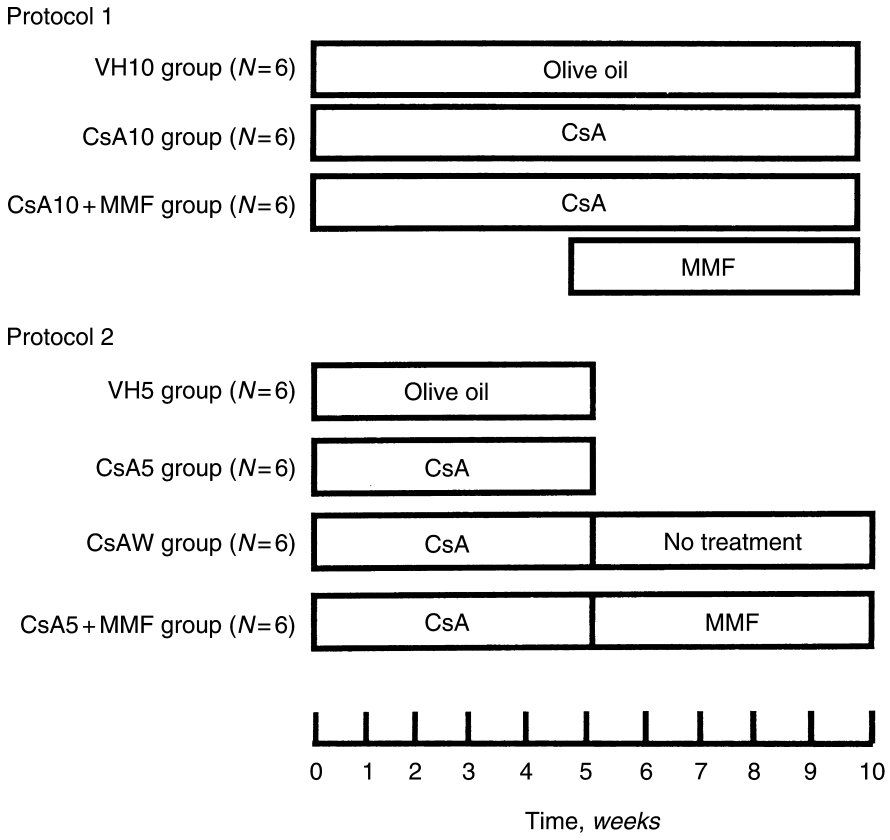

Fig. 1. Experimental design. Two separate studies were performed. The first study was designed to evaluate the effect of combined treatment with cyclosporine A (CsA) and mycophenolate mofetil (MMF) on chronic CsA nephrotoxicity (protocol 1), and the second study was designed to evaluate the effect of CsA withdrawal, and subsequent MMF monotherapy on chronic CsA nephrotoxicity (protocol 2).

eliminates guanosine triphosphate of lymphocytes and monocytes [13]. In addition to immunosuppression, MMF has an anti-inflammatory effect as it inhibits the glycosylation of adhesion molecules [14], osteopontin expression [15], and the recruitment of leukocytes to sites to inflammation. Furthermore, MMF inhibits the production of lymphocyte- and macrophage-derived cytokines and growth factors such as platelet-derived growth factor (PDGF), TGF-β1, interleukin (IL)-2 and tumor necrosis factor (TNF)-α [16]. The anti-inflammatory effect of MMF has been observed in experimental models of chronic uremic rats [17, 18] and ischemia-reperfusion injury [19]. These findings suggest that MMF treatment may be effective in reducing chronic CsA nephropathy by decreasing possible mediators (such as, osteopontin, TGF-β1) in CsA-induced nephrotoxicity.

Recent clinical trials have shown that the addition of MMF and a reduction in the dose of CsA are effective in preventing the progression of CAN [20–23]. While this finding suggests that the CsA dose may be a critical factor in determining graft function in CAN, the effect of MMF on chronic CsA nephropathy has not been examined. Therefore, our study was designed to evaluate the effect of MMF on CsA-induced nephrotoxicity. Chronic CsA nephrotoxicity was produced using a low-salt diet, and the effects of MMF treatment on possible mediators of CsA-induced nephrotoxicity were observed in an experimental model of chronic CsA nephrotoxicity.

METHODS

Animals

Male Sprague-Dawley rats, weighing 225 to 250 g, were housed in individual cases in a temperature- and light-controlled environment in the Catholic University Animal Care Facility. The protocol used in these studies was approved by the Catholic University Animal Care Committee. Rats were received a low salt diet (LSD; 0.05% sodium; Teklad Premier, Madison, WI, USA).

Drugs

Cyclosporine A provided by Novartis Research (East Hanover, NJ, USA) was diluted in olive oil to a final concentration of 7.5 mg/mL. Mycophenolate mofetil (CellCept; Roche Laboratories, Montchair, NJ, USA) was suspended in sterile water by vigorous agitation, with a final concentration of 10 mg/mL. The MMF was given immediately by gavage once daily, 20 mg/kg in a volume of vehicle never exceeding 0.3 mL.

Experimental group

Two separate experiments were conducted as outlined in Figure 1.

Protocol 1. This study was designed to evaluate the combined effect of MMF and CsA on chronic CsA nephrotoxicity. Animals were divided into three groups.

(*1*) VH10 (vehicle) group: Rats received subcutaneous injection of olive oil (1 mg/kg) for 10 weeks ($N = 6$).

(*2*) CsA10 group: Rats received CsA (7.5 mg/kg) for 10 weeks ($N = 6$).

(*3*) CsA10 + MMF group: Rats received CsA for five weeks and then MMF (20 mg/kg) was administered by gavage five weeks later ($N = 6$).

Protocol 2. The second study was designed to evaluate the effects of withdrawal of CsA and subsequent MMF monotherapy in chronic CsA nephrotoxicity.

(*1*) VH5 group: Rats received a daily subcutaneous injection of olive oil (1 mg/kg) for five weeks ($N = 6$).

(*2*) CsA5 group: Rats received a daily subcutaneous injection of CsA (7.5 mg/kg) for five weeks ($N = 6$).

(*3*) CsAW group: Rats received daily subcutaneous injections of CsA (7.5 mg/kg), and then CsA was withdrawn for five weeks ($N = 6$).

(*4*) CsA5+MMF group: Rats received daily subcutaneous injection of CsA (7.5 mg/kg), and were then administered MMF (20 mg/kg) after CsA withdrawal for five weeks ($N = 6$).

These dosages and routes of administration for CsA [24] and MMF [14] were chosen based on previous data.

Basic protocol

After one week of the LSD, weight-matched rats were randomly assigned to the different treatment groups. Daily body weights were recorded. After each treatment period, the systolic blood pressure was measured with a plethysmography using a tail manometer-tachometer system (BP-2000; Visitech Systems, Apex, NC, USA), and 24-hour urine samples were collected in metabolic cages (Nalge Co., Rochester, NY, USA). The following day animals were anesthetized with ketamine and a blood sample and tissue specimens were obtained.

Functional studies

Urinary and serum creatinine was measured by a Cobas autoanalyzer (Roche Diagnostics, Div. Hoffman-La Roche Inc., Montclair, NJ, USA). Creatinine clearance (C_{Cr}) was calculated using a standard formula. The whole blood CsA concentrations were measured by monoclonal radioimmunoassay (Incstar Co., Stillwater, MN, USA).

Histology

Tissue samples were fixed in and embedded in wax. Sections 2 to 4 μm were stained with periodic-acid Schiff (PAS) reagent for evaluation of arteriolopathy and with Trichrome for evaluation of tubulointerstitial fibrosis. These histologic findings were evaluated as previously described by others [10]. Arteriolopathy of the afferent arterioles was characterized by expansion of the cell cytoplasm of terminal arteriolar smooth muscle cells by eosinophilic, granular material and semiquantitatively estimated by counting the percentage of juxtaglomerular afferent arterioles with arteriolopathy per total number of juxtaglomerular arterioles available for examination with a ×20 objective, with a minimum of 60 glomeruli per biopsy assessed. Tubulointerstitial fibrosis (TIF) was semiquantitatively estimated using a color image analyzer (Mustek Paragon 800 SP; Macintosh PowerPC 7100, NIH Image v. 1.5) by counting the percentage of injured areas per field. Scores of 0 to 3+ were given as follows: 0 = normal interstitium; 0.5 = <5%; 1.0 = 5 to 15% injury; 1.5 = 16 to 25% injury; 2.0 = 26 to 35% injury; 2.5 = 36 to 45% injury; 3.0 = >45% injury.

Tdt-mediated dUTP-biotin nick end labeling method (TUNEL assay)

Cells undergoing apoptosis were identified using the ApopTag in situ apoptosis detection kit (Oncor, Gaithersburg, MD, USA). The sections were dewaxed and treated with proteinase K, then incubated with equilibration buffer in a humidified chamber for 10 minutes at room temperature, followed by incubation with working-strength TdT enzyme solution in a humidified chamber at 37°C for two hours. The reaction was terminated by incubation in working-strength stop/wash buffer for 30 minutes at 37°C. After being rinsed with phosphate-buffered saline (PBS), the sections were incubated with anti-digoxigenin peroxidase in a humidified chamber for 30 minutes at room temperature. Sections were then incubated with diaminobenzidine and 0.01% H_2O_2 for five minutes at room temperature. After being rinsed with PBS, the sections were counterstained with hematoxylin and examined by light microscopy. The number of TUNEL-positive cells was quantitated by sum of 20 fields at ×200 magnification [11].

Northern blots for osteopontin and TGF-β1 mRNA

A 1-kb cRNA probe was generated from the 2B7 cDNA clone of rat smooth muscle osteopontin [25]. Sense and antisense cRNA probe were labeled with digoxigenin-uridine triphosphate (DIG-UTP) using a T7 RNA polymerase kit (Boehringer Mannheim GmbH, Mannheim, Germany). Probes were precipitated and incorporation of DIG was determined by dot blotting. Northern blotting was performed as previously reported [26]. Briefly, kidney cortex was homogenized and extracted using the RNAzol reagent (ISO-TEX Diagnostics, USA), and 20 μg samples were denatured with glyoxal and dimethylsulfoxide, size fractionated on 1.2% agarose gels and capillary blotted onto positively-charged nylon membranes (Boehringer). Membranes were hybridized overnight at 68°C or 42°C with DIG-labeled cRNA probe in

a DIG wash and Block Buffer Set solution (Boehringer). Following hybridization, membranes were washed finally in 0.1 × standard sodium citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) at 68°C or 0.2 × SSC/0.1% SDS at 42°C. Bound probes were detected using sheet anti-DIG antibody (Fab) conjugated with alkaline phosphatase (Boehringer) and development with CSPD-star enhanced chemiluminescence (ECL; Boehringer). The densitometry analysis was performed using the NIH Image PC program, with three determinations for each band that were corrected to 18S. To detect TGF-β1 mRNA expression, cDNA probes labeled with ^{32}P-dCTP by random oligonucleotide priming (Boehringer) were used. Data are presented as mean ± SEM.

Immunohistochemistry for osteopontin, renin and ED-1

Sections were dewaxed. Before incubation with primary antibody, the sections were blocked with normal rabbit serum diluted 1:10 in 0.3% bovine serum albumin (BSA) for 30 minutes, and incubated for two hours at 4°C in mouse antiserum against osteopontin (purchased from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA) diluted 1:200 in BSA. The sections were rinsed in Tris-buffered saline (TBS), and incubated for 30 minutes in peroxidase-conjugated rabbit anti-mouse IgG (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA). Sections were incubated with a mixture of 0.05% 3,3′-diaminobenzidine and 0.01% H_2O_2 for five minutes at room temperature, washed with Tris-HCl buffer, counterstained with hematoxylin and examined by light microscopy. The method for ED-1 or renin immunohistochemistry was similar to osteopontin immunohistochemistry. The number of ED-1 positive cell was counted at least in 20 fields of cortex per section under ×200 magnification, and the number of renin-positive glomeruli was counted per 50 glomeruli.

Statistical analysis

Data reported are mean ± SEM, and all statistical analyses were calculated with SYSTAT for Macintosh (v. 5.2; SYSTAT Inc., Chicago, IL, USA). Comparisons between groups were done by analysis of variance (ANOVA) using the Kruskal-Wallis test followed by the Tukey or Dunnet test). The level of statistical significance was $P < 0.05$.

RESULTS

Effect of MMF on body weight, blood pressure and renal function in chronic CsA nephrotoxicity

During study period there were no deaths and no MMF-related gastrointestinal symptoms (such as diarrhea, poor oral intake). Table 1 shows the summary of body weight, systolic blood pressure and whole blood CsA levels in the different groups. Body weight was significantly decreased in the CsA-treated rats compared with the VH-treated rats. No further decrease of body weight was observed with addition of MMF. The CsA withdrawal significantly increased body weight compared with CsA5 group, but MMF monotherapy after CsA withdrawal did not decrease body weight. Systolic blood pressure measured by tail-cuff pressure was not significantly different among study groups, and whole blood CsA levels were not significantly different between CsA10 and CsA10+MMF groups.

Table 1. Body weight, blood pressure and CsA blood levels in different groups

Group	BW *gram*	SBP *mm Hg*	CsA level *ng/mL*
VH10	364 ± 21	124 ± 12	N.C.
CsA10	323 ± 29[a]	120 ± 10	2291 ± 238
CsA10 + MMF	336 ± 10[a]	118 ± 6	2100 ± 199
VH5	319 ± 20	122 ± 11	N.C.
CsA5	295 ± 23[b]	120 ± 10	2095 ± 125
CsAW	343 ± 29[b,c]	127 ± 10	N.C.
CsA5 + MMF	360 ± 10[b,c]	118 ± 6	N.C.

Data are mean ± SEM, $N = 6$ in all groups. Abbreviations are: BW, body weight; SBP, systolic blood pressure; CsA, cyclosporine; MMF, mycophenolate mofetil; VH, vehicle; N.C., not checked.
[a] $P < 0.05$ vs. VH10 group
[b] $P < 0.05$ vs. VH5 group
[c] $P < 0.05$ vs. CsA5 group

Figure 2 shows renal function in the experimental groups. Ten weeks of treatment with CsA significantly reduced the C_{Cr} levels (0.20 ± 0.03 vs. 0.45 ± 0.05 mL/min per 100 g) and increased serum creatinine (S_{Cr}) levels (1.05 ± 0.16 vs. 0.49 ± 0.06 mg/dL) compared with the VH10 group ($P < 0.05$). The addition of MMF (CsA10+MMF group) did not improve the S_{Cr} (1.01 ± 0.15 mg/dL) or C_{Cr} (0.22 ± 0.05 mL/min per 100 g) compared with the CsA 10 group ($P > 0.05$).

The CsAW group showed a significant decrease in S_{Cr} (0.95 ± 0.13 vs. 0.70 ± 0.08 mg/dL; $P < 0.05$) and an increase of C_{Cr} (0.24 ± 0.07 vs. 0.34 ± 0.03 mL/min per 100 g; $P < 0.05$) compared with the CsA5 group. However, withdrawal of CsA and subsequent MMF treatment did not improve either parameter compared with the CsA5 groups ($P > 0.05$).

Effect of MMF on arteriolopathy and interstitial fibrosis in chronic CsA nephrotoxicity

The histology in CsA-treated rats showed typical afferent arteriolopathy. The smooth muscle cells the afferent glomerular arteriole were replaced by a PAS-positive material, resulting in a typical circumferential appearance of the lesion (Fig. 3B). Using a semiquantitative score, the arteriolopathy was significantly higher in the

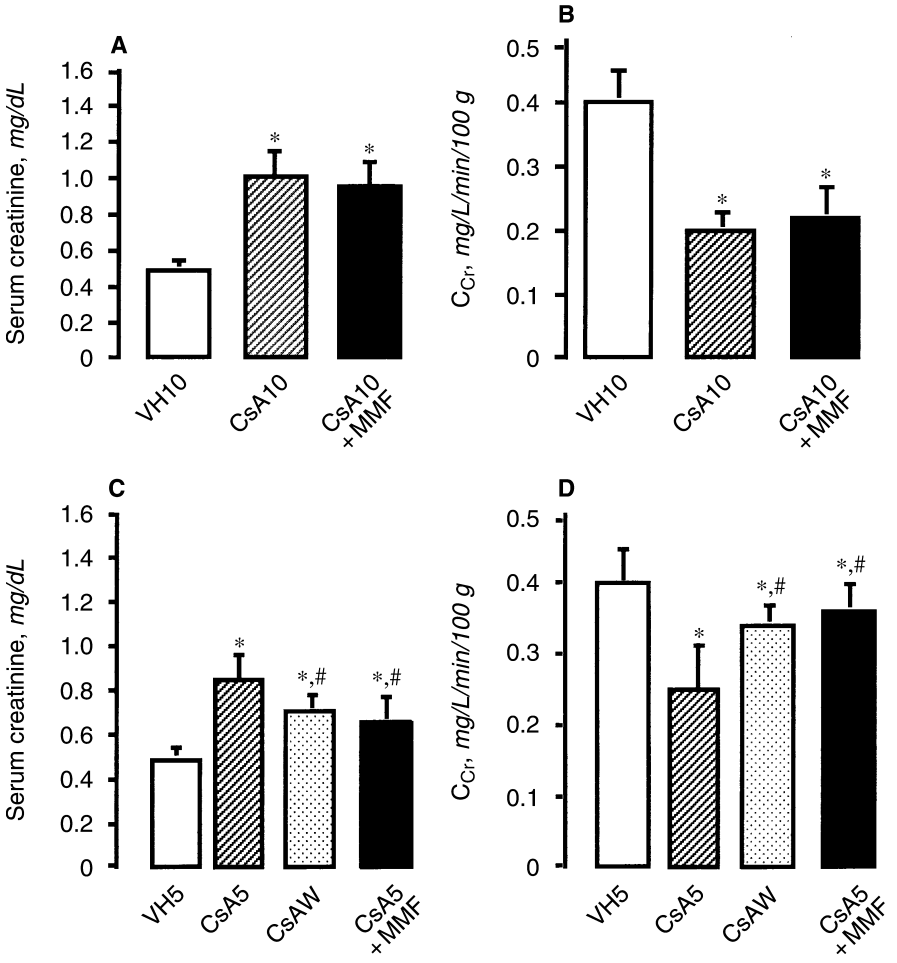

Fig. 2. Effects of MMF treatment on renal function in chronic CsA nephrotoxicity. Note that there is no significant difference in serum creatinine (S_{Cr}) and creatinine clearance (C_{Cr}) between the CsA10 and CsA10+MMF groups (*A*, *B*), and the CsAW and CsA5 + MMF (*C*, *D*) groups. *$P < 0.05$ vs. VH5 or VH10; #$P < 0.05$ vs. CsA5.

CsA10 group than the VH10 group (Table 2). The addition of MMF did not decrease the arteriolopathy score compared with the CsA10 group. In the CsAW group, there was a significant decrease in the arteriolopathy score compared with the CsA5 group, and a further decrease was observed with the administration of MMF after CsA withdrawal.

The histology in CsA-treated rats showed TIF, inflammatory cell infiltration and tubular atrophy (Fig. 4). When the extent of changes in TIF was graded using a 0 to +4 semiquantitative score, the addition of MMF did not decrease the TIF score compared with the CsA10 group (2.1 ± 0.5 vs. 2.3 ± 0.6; $P > 0.05$). In the CsAW group, there was a significant decrease in the TIF score compared with the CsA5 group (1.5 ± 0.4 vs. 2.0 ± 0.6; $P < 0.05$), and a further decrease was observed with the administration of MMF after CsA withdrawal (vs. 1.0 ± 0.3; $P < 0.05$; Fig. 5).

Effect of MMF on apoptotic cell death in chronic CsA nephrotoxicity

Figure 6 shows TUNEL-staining in different experimental groups and Table 2 shows the quantitative analysis of TUNEL-positive cells. In the VH group, TUNEL-positive cells were rarely observed, but their numbers increased in the CsA-treated rat kidneys. In the CsA10 + MMF group, there was no significant decrease in the number of TUNEL-positive cells compared with the CsA10 group. There was a significant decrease in TUNEL-positive cells in the CsAW group (Fig. 6C) compared with the CsA5 group, and MMF treatment after CsA withdrawal further reduced the number of TUNEL-positive cells (Fig. 6D).

Effect of MMF on intrarenal renin expression in chronic CsA nephrotoxicity

Figure 7 shows the intrarenal expression of renin in different experimental groups. Intrarenal expression of renin was minimal in the VH group, but this immunoreactivity and the number of renin-positive glomeruli increased in the kidneys with CsA treatment. Quantitative analysis of renin-positive glomeruli in rat kidney (Table 2) revealed significant increase in the number of renin-positive glomeruli in the CsA10 group compared with the VH group. The addition of MMF (CsA10 +

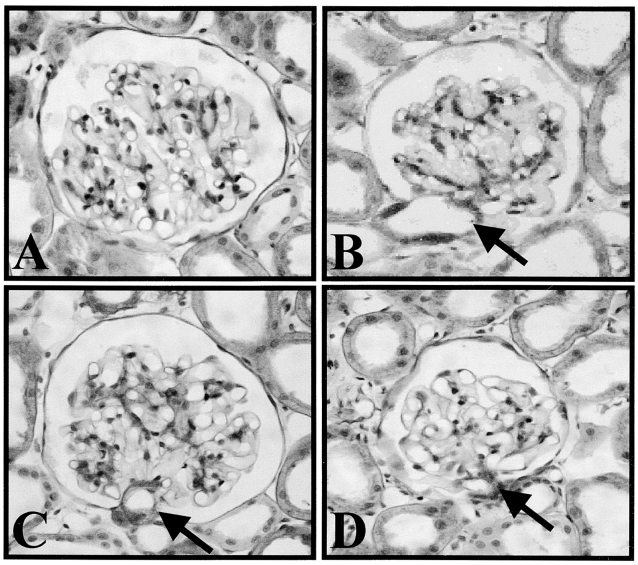

Fig. 3. Effect of MMF treatment on arteriolopathy in chronic CsA nephrotoxicity. (*A*) VH5 group showing a normal glomerulus. (*B*) CsA5 group with the glomerulus showing expansion of the cell cytoplasm of terminal arteriolar smooth muscle cells by eosinophilic, granular material (arrow). (*C*) CsAW group with the glomerulus showing regression of arteriolar hyalinization. (*D*) CsA5 + MMF group with the glomerulus showing almost absence of arteriolar hyalinization. (PAS stain, magnification ×400).

Table 2. Arteriolopathy, number of TUNEL-positive cells and renin-positive glomeruli in different groups

Group	Arteriolopathy %	TUNEL-positive cells	Renin-positive glomeruli
VH10	5 ± 2	2 ± 2	11 ± 3
CsA10	59 ± 13[a]	54 ± 15[a]	25 ± 2[a]
CsA10 + MMF	51 ± 12[a]	52 ± 17[a]	22 ± 2[a]
VH5	4 ± 3	3 ± 2	10 ± 4
CsA5	48 ± 19[b]	45 ± 14[b]	21 ± 3[b]
CsAW	32 ± 8[b,c]	28 ± 15[b,c]	17 ± 4[b,c]
CsA5 + MMF	20 ± 6[b,d]	18 ± 7[b,d]	13 ± 3[b,c,d]

Data are mean ± SEM, $N = 6$ in all groups. Arteriolopathy was semiquantitatively measured by counting the percentage of juxtaglomerular afferent arterioles with arteriolopathy per total number of juxtaglomerular arterioles available for examination with ×20 objective, with a minimum of 60 glomeruli per biopsy. The number of apoptosis positive cells per 20 fields was determined using the TUNEL assay at a ×200 magnification. The number of renin-positive glomeruli are taken from a universe of 50 glomerui examined.

[a] $P < 0.05$ vs. VH10
[b] $P < 0.05$ vs. VH5
[c] $P < 0.05$ vs. CsA5
[d] $P < 0.05$ vs. CsAW

MMF group) did not reduce the number of renin-positive glomeruli; however, withdrawal of CsA significantly decreased the number of renin-positive glomeruli (Fig. 7C) compared with the CsA5 group, and a further decrease was observed with MMF treatment (Fig. 7D).

Effect of MMF on macrophage infiltration in chronic CsA nephrotoxicity

Figure 8 shows the ED-1-positive cells in CsA-treated rat kidneys. ED-1–positive cells were minimal in the VH

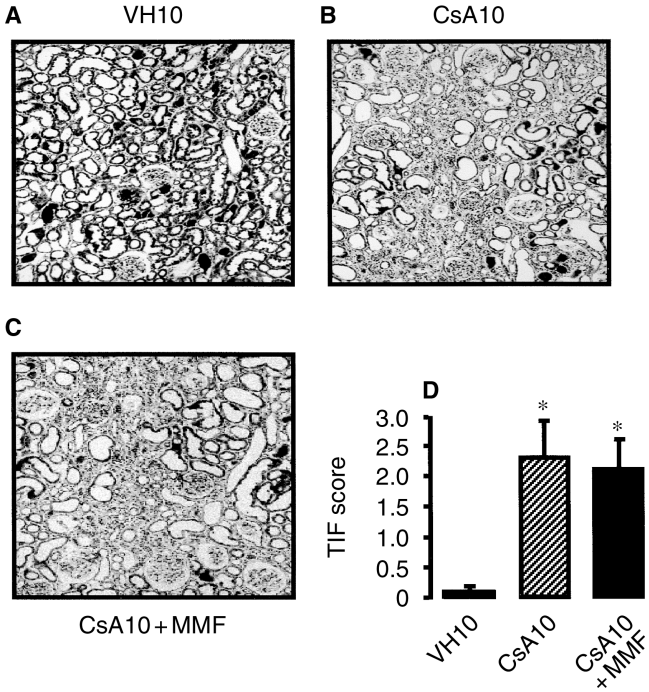

Fig. 4. Effect of the combined treatment with CsA and MMF on interstitial fibrosis in established chronic CsA nephrotoxicity. CsA treatment for 10 weeks produced interstitial fibrosis, inflammatory cells infiltration, and tubular atrophy. Note there is no significant improvement of tubulointerstitial fibrosis (TIF) with the addition of MMF in chronic CsA nephrotoxicity (Trichrome stain, magnification ×100; *$P < 0.05$ vs. VH10).

group (3 ± 2), but their number increased with CsA treatment for 10 weeks (22 ± 5; $P < 0.05$). Simultaneous administration of MMF and CsA slightly decreased ED-1–positive cells, though this was not statistically significant (18 ± 6; $P > 0.05$). In the CsA withdrawal group, ED-1–positive cells were decreased significantly as compared with the CsA5 group (17 ± 3 vs. 13 ± 4, $P < 0.05$), and further decrease of ED-1-positive cells was observed with MMF treatment in the CsA-withdrawn state (vs. 7 ± 2, $P < 0.05$; Fig. 9).

Effect of MMF on osteopontin expression in chronic CsA nephrotoxicity

Northern blot and immunohistochemical analyses were used to assess osteopontin expression. In the CsA10 group, there was 3.7-fold increase in osteopontin mRNA expression compared with the VH10 group. The addition of MMF did not decrease osteopontin expression (Fig. 10). The CsAW group showed a decrease in osteopontin mRNA expression compared with the CsA5 group (2.5- vs. 1.8-fold, respectively, compared with the VH group; $P < 0.05$), and the CsA5 + MMF group showed a further decrease compared with the CsAW group (1.3-fold relative the VH group; Fig. 11). Minimal immunoreactivity for osteopontin was evident in the VH-treated rat kidney, but the extent and immunoreactivity of osteo-

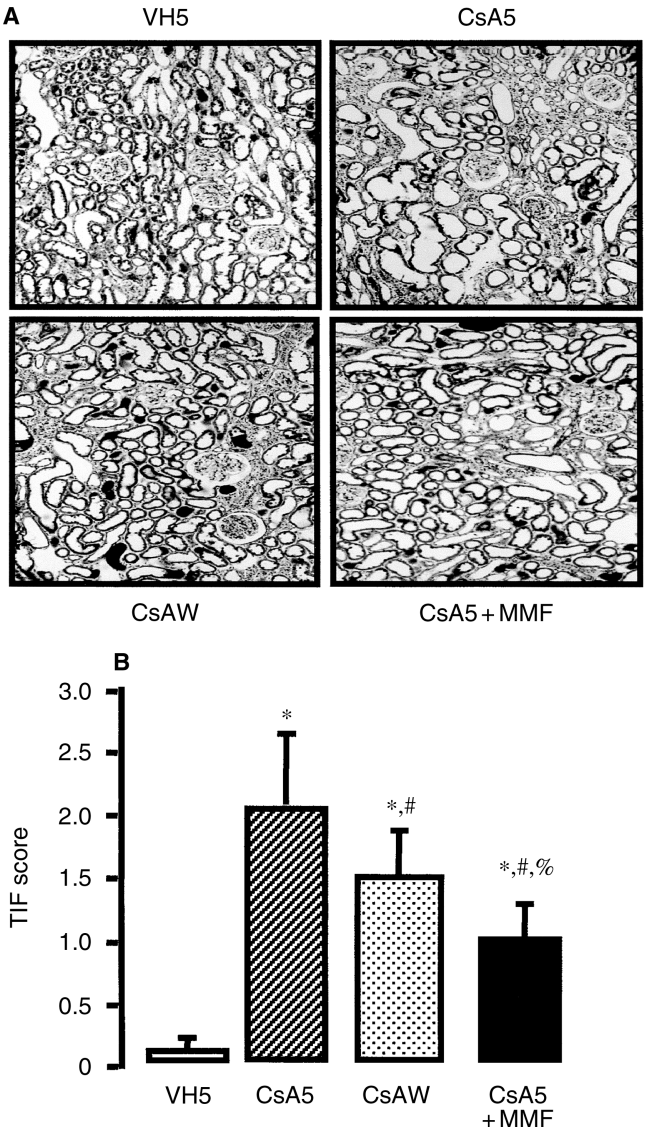

Fig. 5. Effect of CsA withdrawal or MMF administration on tubulointerstitial fibrosis (TIF) in established chronic CsA nephrotoxicity. Treatment with CsA for five weeks resulted in a pathology typical of chronic CsA nephrotoxicity. With CsA withdrawal, interstitial fibrosis decreased compared with the CsA5 group, and a further decrease in interstitial fibrosis was observed with MMF treatment after CsA withdrawal. (Magnification, ×100; *$P < 0.05$ vs. VH5; #$P < 0.05$ vs. CsA5; %$P < 0.05$ vs. CsAW).

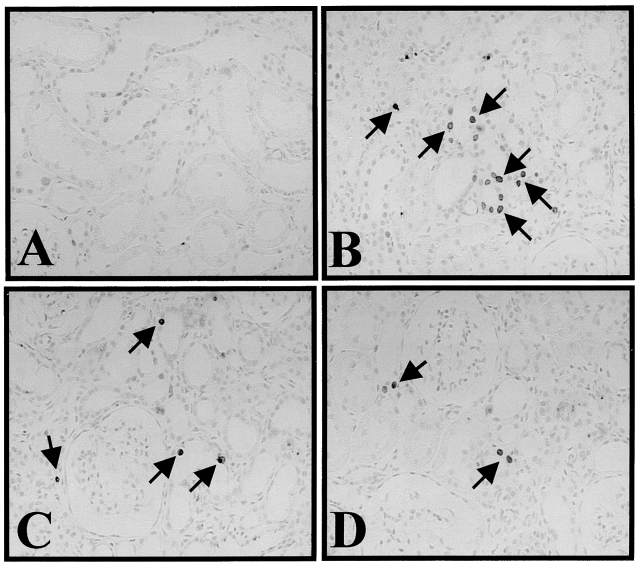

Fig. 6. Effect of MMF on apoptotic cell death detected by TUNEL-staining in chronic CsA nephrotoxicity. (*A*) VH5 group. Note the lack of TUNEL-positive cells. (*B*) CsA5 group. Note the significant increase in TUNEL-positive cells (arrows) in chronic CsA nephrotoxicity. (*C*) CsAW group. Note the decrease in TUNEL-positive cells. (*D*) CsA5 + MMF group. Note the further decrease of TUNEL-positive cells with MMF treatment after CsA withdrawal (magnification, ×200).

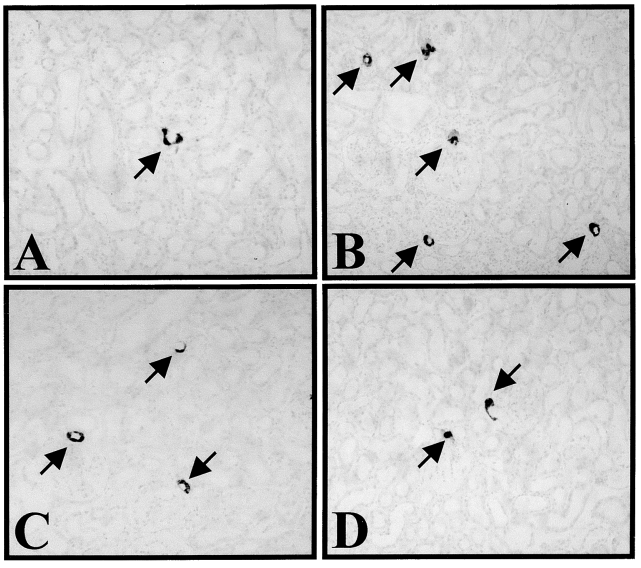

Fig. 7. Effect of MMF on intrarenal renin expression in chronic CsA nephrotoxicity. (*A*) The VH5 group shows a minimal immunoreactivity of renin. (*B*) The CsA5 group has a significantly increased immunoreactivity and extent of renin-positive glomeruli (arrows). (*C*) The CsAW group has reduced renin-positive glomeruli (arrows). (*D*) The CsA5 + MMF group has a further decrease of renin-positive glomeruli (arrows) with MMF treatment after CsA withdrawal (magnification, ×200).

pontin protein increased with CsA treatment, and was observed in injured tubules and interstitial areas. Simultaneous administration of CsA and MMF did not cause a decrease in immunoreactivity compared with the CsA5 group, but such a decrease was evident in the CsAW and CsA5 + MMF groups.

Effect of MMF on TGF-β1 mRNA expression in chronic CsA nephrotoxicity

Cyclosporine A treatment for 10 weeks caused a 3.9-fold increase in TGF-β1 mRNA expression compared with the VH10 group, but the addition of MMF did not decrease this expression. With CsA withdrawal, TGF-β1 mRNA expression was significantly decreased compared with the CsA5 group (2.9- vs. 2.3-fold, respectively; $P <$

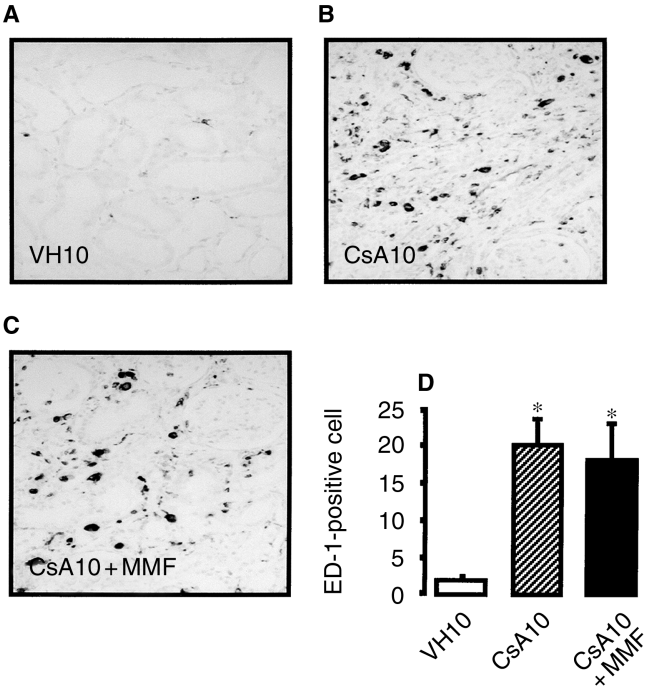

Fig. 8. Effect of MMF on macrophage infiltration in chronic CsA nephrotoxicity. The CsA10 group showed a significant increase of ED-1–positive cells compared with the VH group. Note that there is no significant decrease in ED-1-positive cells with the addition of MMF in established chronic CsA nephrotoxicity (magnification, ×200; *$P <$ 0.05 vs. VH10).

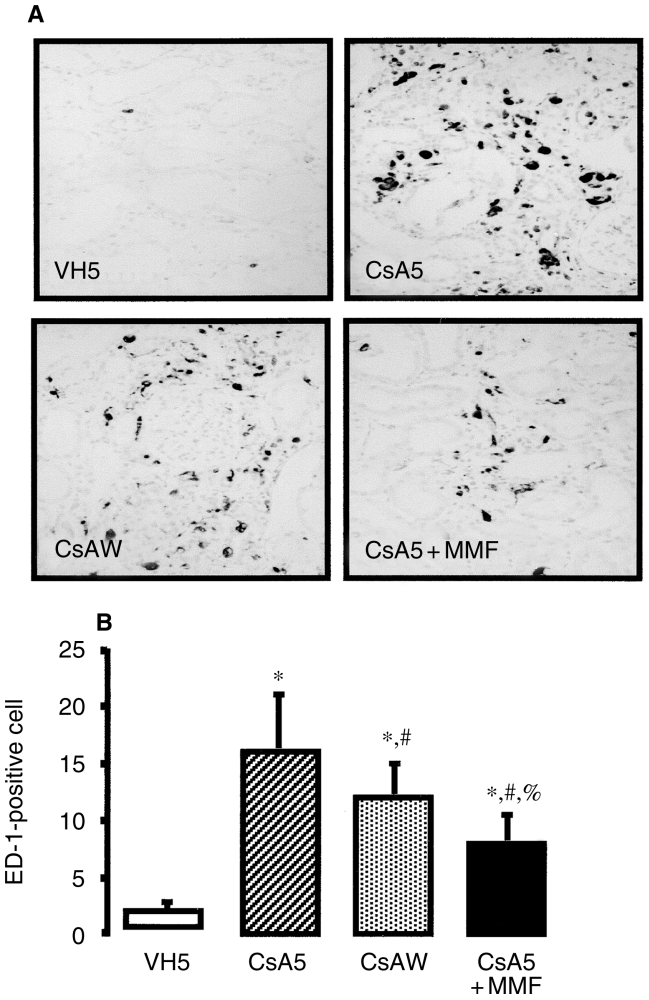

Fig. 9. Effect of CsA withdrawal and MMF monotherapy on macrophage infiltration in chronic CsA nephrotoxicity. (*A*) Immunohistochemical analysis of ED-1. Note the increased immunoreactivity in the CsA-treated rat kidney. (*B*) Quantitative analysis of ED-1–positive cells in the experimental groups. Note that ED-1–positive cells are increased in the CsA5 group, and decreased in the CsAW and CsA5+MMF groups (magnification, ×200; *$P <$ 0.05 vs. VH5; #$P <$ 0.05 vs. CsA5; %$P <$ 0.05 vs. CsAW).

0.05), and a further decrease was observed with MMF treatment (1.8-fold; $P < 0.05$; Fig. 12).

DISCUSSION

The results of our study clearly show that concurrent administration of MMF and CsA is negligibly effective in preventing the development chronic CsA nephrotoxicity, but MMF monotherapy as well as withdrawal of CsA is effective in inhibiting its progression. The results of our study clarify the roles of MMF or CsA in the progression of chronic CsA nephrotoxicity, and provide a rationale for MMF use in chronic CsA nephrotoxicity.

It is well known that MMF inhibits the proliferation of resident renal cells [27, 28] and collagen deposition [29]. Furthermore, MMF inhibits the production of lymphocyte- and macrophage-derived TGF-β1, TNF-α, and IFN-γ in specific experimental models [16, 30]. Therefore, considering the variety of potentially beneficial actions attributed to MMF, we expected MMF to have a protective effect on CsA-induced nephrotoxicity. However, our study demonstrated no beneficial effects from the administration of MMF in inhibiting chronic CsA nephrotoxicity. Similar results were reported from a human study [abstracts; Schurter et al, *Am Soc Transplant Physicians Abstract Book* 16:133 (196A), 1997; and Smith et al, *Am Soc Transplant Physicians Abstract Book* 16:134 (197A), 1997]. The addition MMF to prednisone-CsA therapy without a reduction of in the CsA dose conferred no significant benefit on patients with CAN. These findings suggest that the anti-inflammatory action of MMF is not strong enough to overcome CsA-induced nephrotoxicity when the kidney is continuously exposed to CsA. Therefore, the dose of CsA may be one of the factors in determining graft function in CAN.

The effect of CsA withdrawal on the progression of chronic CsA nephrotoxicity depends upon the accumulated CsA dose and the timing of CsA withdrawal [31]. The results of our study revealed that CsA withdrawal significantly improved not only renal function, but also histologic parameters (apoptotic cell death, inflamma-

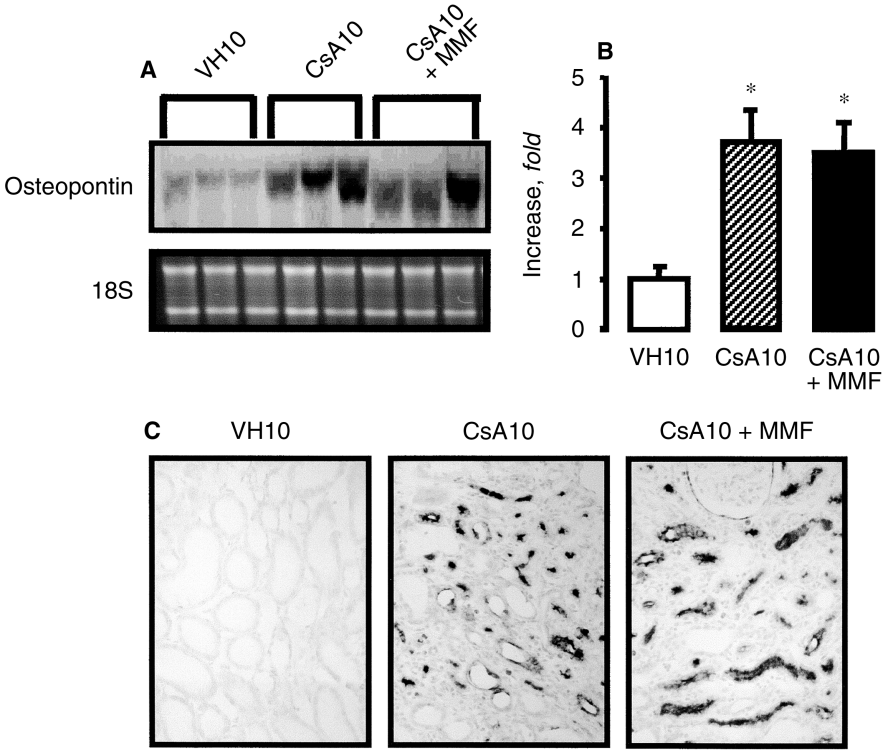

Fig. 10. Effect of combined treatment with CsA and MMF on osteopontin mRNA and protein expression. (*A*) Northern blot analysis for osteopontin mRNA. (*B*) Relative expression of osteopontin mRNA. (*C*) Immunohistochemical analysis of osteopontin. Note the significant increase of osteopontin mRNA and protein expression the CsA10 group. However, there was no significant difference in osteopontin expression between the CsA10 and CsA10 + MMF groups (magnification, ×200; $*P < 0.05$ vs. VH10).

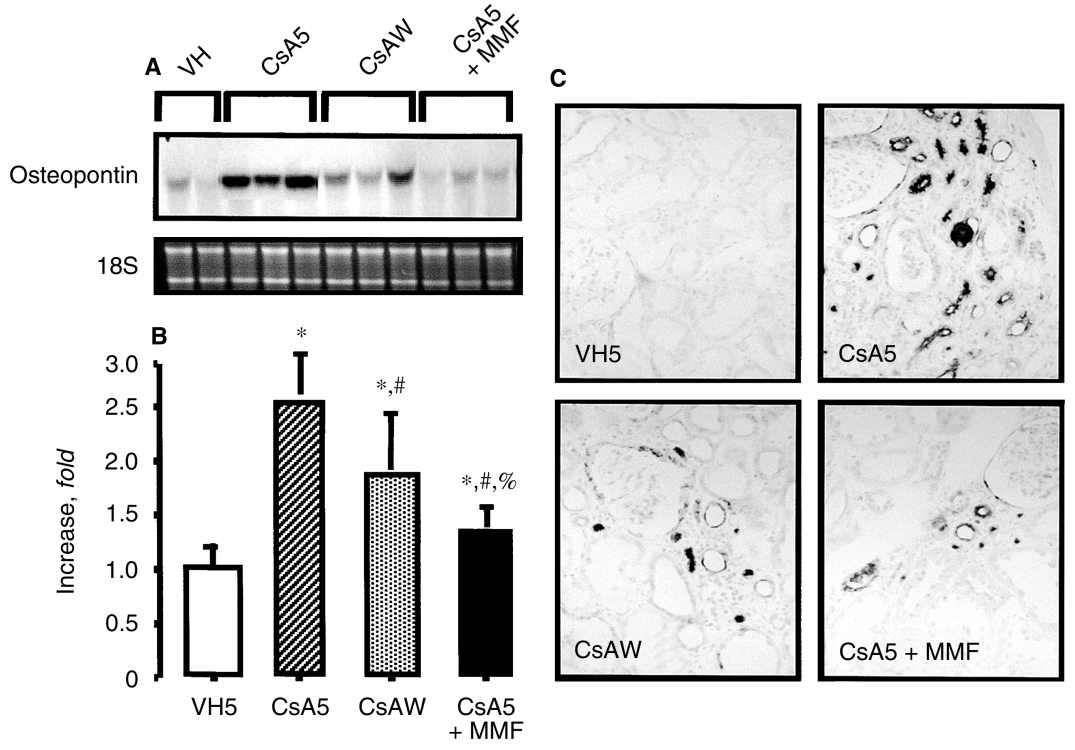

Fig. 11. Effect of CsA withdrawal or MMF monotherapy on osteopontin mRNA and protein expression in chronic CsA nephrotoxicity. (*A*) Northern blot analysis of osteopontin mRNA. (*B*) Relative expression of osteopontin mRNA. (*C*) Immunohistochemical analysis of osteopontin. Note the significant increase in osteopontin mRNA or protein in CsA-treated rat kidney. With CsA withdrawal, osteopontin mRNA expression decreased significantly and a further decrease was observed with MMF treatment after CsA withdrawal (magnification, ×200; $*P < 0.05$ vs. VH5; $\#P < 0.05$ vs. CsA5; $\%P < 0.05$ vs. CsAW).

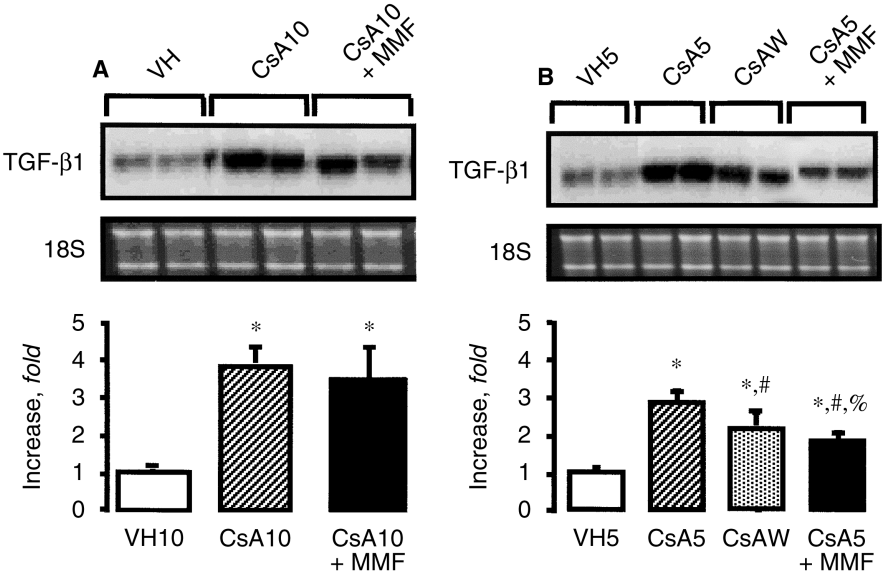

Fig. 12. Effect of MMF treatment on TGF-β1 mRNA expression in chronic CsA nephrotoxicity. (A) The combined effect of CsA and MMF on TGF-β1 mRNA expression in established chronic CsA nephrotoxicity. Note the significant increase of TGF-β1 mRNA expression in CsA-treated rat kidney, but no significant decrease of TGF-β1 mRNA expression with addition of MMF. (*B*) Effect of CsA withdrawal or MMF monotherapy on TGF-β1 mRNA expression in chronic CsA nephrotoxicity. Note the significant decrease of TGF-β1 mRNA expression with CsA withdrawal as compared with the CsA group, and further decrease of TGF-β1 mRNA expression with MMF treatment in the CsA-withdrawal state (*$P < 0.05$ vs. VH5 or VH10; #$P < 0.05$ vs. CsA5; %$P < 0.05$ vs. CsAW).

tory cell infiltration, arteriolopathy and TIF) in rat kidneys. At the molecular level, histologic improvement was accompanied by decreased expression of renin-positive glomeruli, osteopontin and TGF-β1 mRNAs. This finding is consistent with human studies that described the effects of reduction or withdrawal of CsA on CAN (abstracts; Wei et al, *J Am Soc Nephrol* 10:92A, 1999; Song et al, *Transplantation* 67:S236, 1999). Based on above findings, we suggest that the mechanism underlying the benefits of CsA withdrawal is associated with a reduction of RAS activity and a decrease in the profibrogenic cytokine (TGF-β1) or proinflammatory cytokines (osteopontin), which play an important role in the pathogenesis of chronic CsA nephrotoxicity [9, 10].

The result of this study indicates that the MMF treatment in the CsA-withdrawal state decreases chronic CsA nephrotoxicity. In evaluating possible mechanisms responsible for the protection offered by MMF, we first suggest that the reduction of tubulointerstitial damage by MMF may be a consequence of the suppression of the local inflammatory reaction that is fueled by the infiltration of inflammatory cells [13, 18, 32]. In the present study, increased ED-1–positive cells and up-regulated expression of osteopontin and TGF-β1 mRNAs in CsA-treated rat kidneys were significantly decreased with MMF treatment after CsA withdrawal. This finding suggests that MMF itself may attenuate tubulointerstitial inflammation and fibrosis by inhibiting pro-inflammatory and pro-fibrogenic cytokines [33].

It also is possible that MMF treatment inhibits the renin-angiotensin system (RAS) in CsA-treated rat kidney. Activation of the RAS is associated with the pathogenesis of chronic CsA nephrotoxicity [5], and blocking this system with enalapril or losartan prevents chronic CsA nephrotoxicity [34]. Until now, the influence of MMF on the RAS has not been fully understood, but two recent studies revealed that MMF treatment decreased angiotensin II-positive cells among infiltrating mononuclear cells in an animal model of salt-sensitive hypertension [35, 36]. Our current study evaluated intrarenal renin expression using immunohistochemistry, and quantitative analysis revealed that MMF treatment after CsA withdrawal as well as CsA withdrawal alone significantly decreased the number of renin-positive glomeruli in chronic CsA nephrotoxicity. On the basis of our study and a previous report, we propose that that renoprotective effect of MMF on chronic CsA nephrotoxicity may be related, in part, to the decreased activity of the RAS.

Reports have shown that MMF is effective in reducing CAN when the CsA dose is reduced to 50% [20], or low trough levels (40 to 60 ng/mL) [21], or even after CsA withdrawal [23]. These findings suggest that CsA-induced nephrotoxicity potentially influences CAN, and provides a rationale for the reduction or withdrawal of CsA and the use of non-nephrotoxic immunosuppressants such as MMF and rapamycin in current immunosuppression regimens [37, 38]. This strategy involves the reduction of CsA-induced nephrotoxicity and the maximization of immunosuppression with non-nephrotoxic drugs. In our study, the effects of MMF on rats treated with reduced CsA dose were not evaluated, but we postulate that a reduction in the CsA dose may have beneficial effects on renal function and histology in CsA-induced nephrotoxicity. Furthermore, enhanced immunosuppression with MMF may be beneficial in clinical practice. Further study is required to evaluate the appropriate doses of CsA and MMF to achieve optimal immunosuppression while avoiding the deterioration in graft function caused by CsA.

In summary, our present study clarifies the roles of CsA and MMF in the progression of chronic CsA neph-

rotoxicity. A combined treatment with MMF and CsA is not effective; however, MMF monotherapy after CsA withdrawal is effective in preventing the progression of chronic CsA nephrotoxicity. This finding provides a rationale for MMF treatment in chronic CsA nephrotoxicity.

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